β-Amino Acid Analogs of an Insect Neuropeptide Feature Potent Bioactivity and Resistance to Peptidase Hydrolysis

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ABSTRACT:

Insect neuropeptides of the insect kinin class share a common C-terminal pentapeptide sequence $F^1X_1^2X_2^3W^4G^5$-NH_2 (X_2^3 = P, S, A) and regulate such critical physiological processes as water balance and digestive enzyme release. Analogs of the insect kinin class, in which the critical residues of F^1, P^3, and W^4 were replaced with β^3-amino acid or their β^2-homo-amino acid variants, have been synthesized by the solid phase peptide strategy. The resulting single- and double-replacement analogs were evaluated in an insect diuretic assay and enzyme digestion trials. Analogs modified in the core P^3 position produce a potent and efficacious diuretic response that is not significantly different from that obtained with the endogenous achetakinin peptides. The analogs also demonstrate enhanced resistance to hydrolysis by ACE and NEP, endopeptidases that inactivate the natural insect neuropeptides. This paper describes the first instance of β-amino acids analogs of an arthropod peptide that demonstrate significant bioactivity and resistance to peptidase degradation. ©2006 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 88:76–82, 2007.

Keywords: malphigian tubule; cricket; Acheta domesticus; β-amino acids; peptides; insect kinin; neuropeptide; neprilysin, NEP; angiotensin converting enzyme, ANCE

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INTRODUCTION

Insect neuropeptides of the insect kinin class share a common C-terminal pentapeptide sequence Phe^1-Xaa_1^2-Xaa_2^3-Trp^4-Gly^5-NH_2 (Xaa_1^2 = His, Asn, Phe, Ser, or Tyr; Xaa_2^3 = Pro, Ser, or Ala). They have been isolated from a number of insects, including species of Dictyoptera, Lepidoptera, and Orthoptera. The first members of this insect neuropeptide family were isolated on the basis of their

ability to stimulate contractions of the isolated cockroach hindgut,[1–3] but they are also potent diuretic peptides that stimulate the secretion of primary urine by Malpighian tubules, organs involved in the regulation of salt and water balance.[4–6] More recently, insect kinins, or analogs, have been reported to inhibit weight gain by larvae of the tobacco budworm (*Heliothis virescens*) and corn earworm (*Helicoverpa zea*),[3,7] both serious agricultural pests.

Structurally, the insect kinins require an intact C-terminal pentapeptide sequence for full cockroach myotropic and cricket diuretic activity, which therefore represents the active core.[8] Evaluation of an Ala-replacement analog series of the insect kinin active core region confirms the importance of the Phe and Trp sidechains, because these are the only two replacements that lead to complete loss of myotropic and diuretic activity.[8] The variable position 2 tolerates wide variations of side chain character, although aromatic residues promote highest potencies in Malpighian tubule fluid secretion assays.[8] It has been shown that the natural Achetakinins elicit cricket Malphighian tubule fluid secretion at EC_{50} values ranging from about 18 to 325 p*M*.[4] The active core sequence Phe[1]-Tyr[2]-Pro[3]-Trp[4]-Gly[5]-NH_2 is equipotent with the parent nonapeptide in this assay. Conformational studies on active, restricted conformation analogs indicate that the active conformation adopted by the C-terminal pentapeptide insect kinin core at the cricket Malpighian tubule receptor site is the *cis*Pro type VI β-turn over core residues 1–4.[6,8–11]

Unfortunately, insect kinin peptides are unsuitable as pest control agents and research tools for insect neuroendocrinologists due to susceptibility to both exo- and endopeptidases in the hemolymph and gut of the insect. Members of the insect kinin family are hydrolyzed, and therefore inactivated, by tissue-bound peptidases of insects. Two susceptible hydrolysis sites in insect kinins[3] have been reported. The primary site is between the Pro[3] and the Trp[4] residues, with a secondary site N-terminal to the Phe[1] residue in natural extended insect kinin sequences (Figure 1). Experiments demonstrate that angiotensin converting enzyme (ACE) from the housefly can cleave at the primary hydrolysis site, whereas neprilysin (NEP) can cleave insect kinins at both the primary and secondary hydrolysis sites.[3,10,11,13,14]

Incorporation of β-amino acids can enhance both resistance to peptidase attack and biological activity[15,16] and this strategy has not been previously applied to insect neuropeptides. In this article we describe the synthesis of a number of analogs of the insect kinin C-terminal pentapeptide core in which the critical residues Phe[1] Pro[3] and Trp[4] are replaced with β^3-amino acid or their β^2-homo-amino acid counterparts (Figure 2). Several of these analogs display potent diuretic activity in the cricket *Acheta domesticus* and enhanced resistance to the endopeptidases ACE and NEP, enzymes which deactivate the natural insect kinins. All analogs are also blocked at the N-terminus with an Ac group, which confers resistance to hydrolytic degradation by an additional class of peptidases, the aminopeptidases.[17]

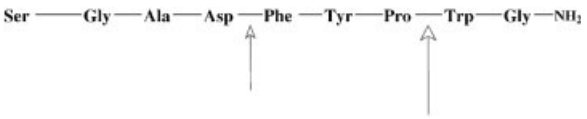

FIGURE 1 Primary (large arrow) and secondary (small arrow) of tissue-bound peptidase hydrolysis sites for the natural cricket insect kinin SGADFYPWG-NH_2 (AK-I).[3,12]

1. Ac-Arg-[**β^3Phe**]-Phe-Pro-Trp-Gly-NH_2 **1457**
2. Ac-Arg-[**β^2homoPhe**]-Phe-Pro-Trp-Gly-NH_2 **1458**
3. Ac-Arg-Phe-Phe-Pro-[**β^3Trp**]-Gly-NH_2 **1459**
4. Ac-Arg-Phe-Phe-Pro-[**β^2Trp**]-Gly-NH_2 **1656**
5. Ac-Arg-Phe-Phe-[**β^3Pro**]-Trp-Gly-NH_2 **1460**
6. Ac-Arg-[**β^3Phe**]-Phe-[**β^3Pro**]-Trp-Gly-NH_2 **1552**
7. Ac-Arg-[**β^3Phe**]-Phe-Phe-[**β^3Pro**]-Trp-Gly-NH_2 **1577**
8. Ac-Arg-Phe-[**β^3Phe-β^3Pro**]-Trp-Gly-NH_2 **1578**

MATERIALS AND METHODS

Peptide Synthesis

Insect kinin analogs were synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Applied Biosystems, Foster City, CA) on an ABI 433A peptide synthesizer (Applied Biosystems) under previously described conditions.[8] Crude products were purified on a Waters C_{18} Sep Pak cartridge and a Delta Pak C_{18} reverse-phase column (8 mm × 100 mm, 15 μm particle size and 100 Å pore size) on a Waters 600 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA) and Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 10% B was followed by the Waters linear program to 90% B over 25 min; flow rate, 2 ml/min. Delta Pak C18 retention times: **1457** (Ac-R[**β^3Phe**]FPWGa), 16.2 min; **1458** (Ac-R[**β^2homoPhe**]FPWGa), 17.5 min; **1459** (Ac-RFFP[**β^3Trp**]Ga), 14.3 min; **1656** (Ac-RFFP[**β^2Trp**]Ga), 15.4 min; **1460** (Ac-RFF[**β^3Pro**]WGa), 15.1 min; **1552** (Ac-R[**β^3Phe**]F[**β^3Pro**]WGa), 16.2 min; **1577** (Ac-R[**β^3Phe**]FF[**β^3Pro**]WGa), 14.1 min; and **1578** (Ac-RF[**β^3Phe-β^3Pro**]WGa), 12.2 min. The peptides were further purified on a Waters Protein Pak I125 column (7.8 mm × 300 mm) (Milligen, Milford, MA). Conditions: Flow rate: 2.0 ml/min; Solvent A = 95% acetonitrile made to 0.01% TFA; Solvent B = 50% aqueous acetonitrile made to 0.01% TFA; 100% A isocratic for 4 min, then a linear program to 100% B over 80 min. WatPro retention time: **1457** (Ac-R

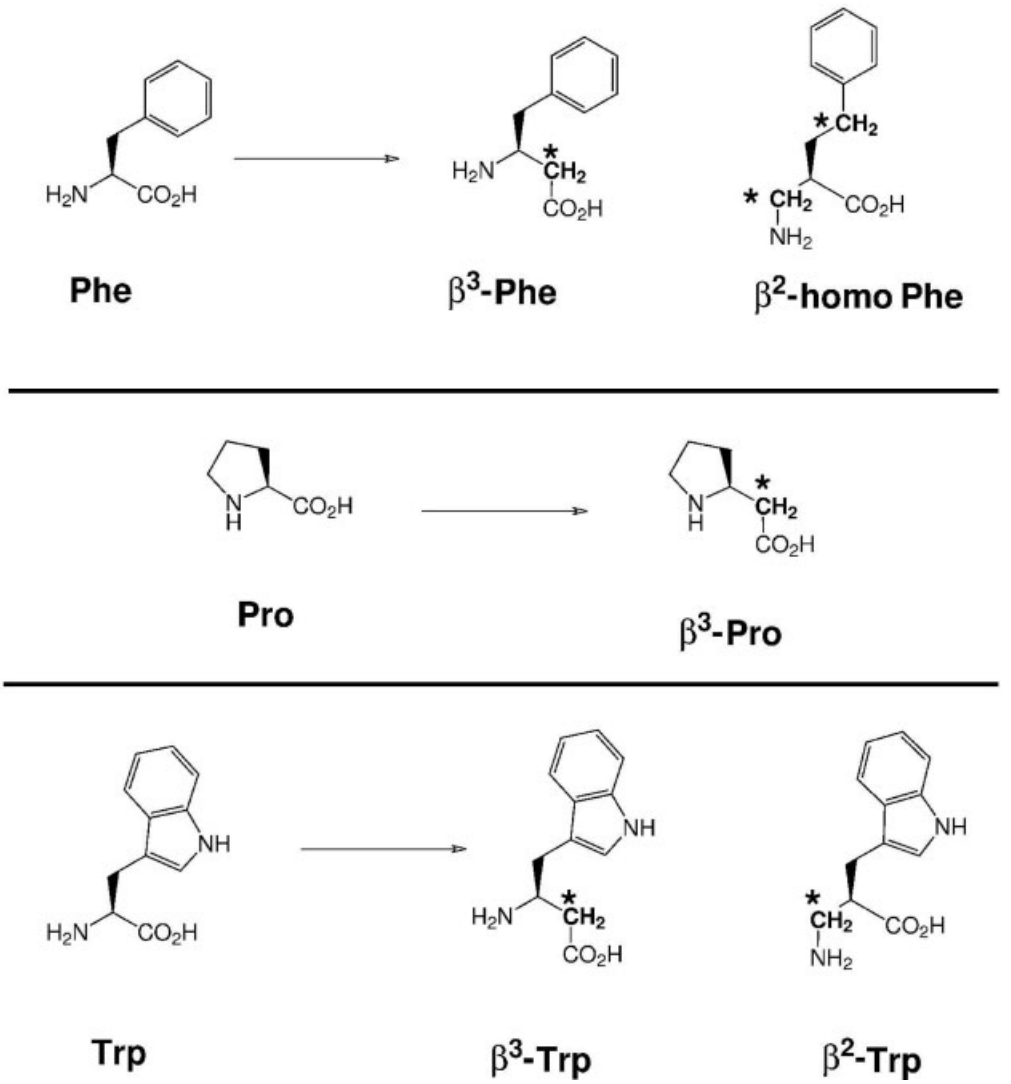

FIGURE 2 β-Amino acid variants[15,16] of natural α-amino acids found in insect kinin neuropeptides. Asterisks identify positions where methylene groups are incorporated.

[***β*³Phe**]FPWGa), 6.2 min; **1458** (Ac-R[***β*² homoPhe**]FPWGa), 6.0 min; **1459** (Ac-RFFP[***β*³Trp**]Ga), 6.4 min; **1656** (Ac-RFFP[***β*²Trp**]Ga), 6.0 min; **1460** (Ac-RFF[***β*³Pro**]WGa), 5.8 min; **1552** (Ac-R[***β*³Phe**]F[***β*³Pro**]WGa), 6.4 min; **1577** (Ac-R[***β*³Phe**]FF[***β*³Pro**]WGa), 5.8 min; and **1578** (Ac-RF[***β*³Phe-*β*³Pro**]WGa), 6.2 min. Amino acid analysis was carried out under previously reported conditions[8] and used to quantify the peptide and to confirm identity, leading to the following analyses: **1457** (Ac-R[***β*³Phe**]FPWGa): F[1.0],G[1.1],P[1.0],R[0.9]; **1458** (Ac-R[***β*²-homoPhe**]FPWGa): F[1.0],G[1.0],P[1.0],R[1.0]; **1459** (Ac-RFFP[***β*³Trp**]Ga): F[2.0],G[1.1],P[1.0],R[0.9]; **1656** (Ac-RFFP[***β*²Trp**]Ga), F[2.0],G[0.9],P[1.0],R[1.0]; **1460** (Ac-RFF[***β*³Pro**]WGa): F[2.0],-G[1.0],R[0.9]; **1552** (Ac-R[***β*³Phe**]F[***β*³Pro**]WGa): F[1.0],G[1.1],-R[1.0]; **1577** (Ac-R[***β*³Phe**]FF[***β*³Pro**]WGa): F[2.0],G[1.0],R[1.2]; **1578** (Ac-RF[***β*³Phe-*β*³Pro**]WGa): F[1.0],G[1.0],R[1.0]. The identity of the peptide analogs were confirmed via MALDI-TOF MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical, Manchester, UK) with the presence of the following molecular ions ($M + H^+$): **1457** (Ac-R[***β*³Phe**]FPWGa), 865.1 (calc. 864 M^+); **1458** (Ac-R[***β*²homoPhe**]FPWGa), 878.7 (calc. 878 M^+); **1459** (Ac-RFFP[***β*³Trp**]Ga), 865.2 (calc. 863 M^+); **1656** (Ac-RFFP[***β*²Trp**]Ga), 865.1 (calc. 864 M^+); **1460** (Ac-RFF[***β*³Pro**]WGa), 865.2 (calc. 864 M^+); **1552** (Ac-R[***β*³Phe**]F[***β*³Pro**]WGa), 879.3 (calc. 878 M^+); **1577** (Ac-R[***β*³Phe**]FF [***β*³Pro**]WGa), 1026.6 (calc. 1025 M^+); **1578** (Ac-RF[***β*³Phe-*β*³Pro**]WGa), 879.2 (calc. 878 M^+).

Cricket Malpighian Tubule Secretion Bioassay

Crickets were reared as described[4] and fed a diet of turkey starter crumbs. Water was provided ad lib. Malpighian tubules were

removed from 6- to 12-day-old adult virgin females. Single tubules were isolated in vitro as described.[4] After 40 min equilibration period, the bathing fluid was changed and the rate of secretion, in picoliters per millimeter length of tubule per minute (pl/mm/min), was determined over 40 min (control rate). Thereafter, the bathing fluid was exchanged for one containing the peptide analog and the rate of secretion was determined over an additional 40 min period (experimental rate). Diuretic activity was calculated as the difference between control and experimental rates and the results are expressed as a percentage of the response obtained with a supermaximal dose (10 n*M*) of achetakinin I assayed alongside the test analogs. All experiments were performed at room temperature (21–24°C).[4]

ACE Trials

Drosophila ACE (M_r 67,000) was purified from a soluble extract of adults as described elsewhere[13,14] and yielded enzyme that appeared as a single band by SDS-PAGE. Unless otherwise stated, peptides (12–750 μM, final concentration) were incubated with 100 ng *Musca* ACE in 100 m*M* Tris-HCl, pH 8.3, in the presence of 10 m*M* $ZnCl_2$ and 0.3*M* NaCl at 37°C. Reactions (15 μl) were terminated after 2 h either by heating to 100°C for 5 min or by the addition of 5 μl of 8%(v/v) TFA, followed by centrifugation (13,000*g*, Microcentaur) for 2 min and diluted to 100 μl with 0.1% (v/v) TFA before HPLC analysis.

HPLC analysis was performed using a Pharmacia SuperPac Pep-S column (250 × 4 mm^2, internal diameter) packed with 5 μm ODS particles. The column was eluted with 5% acetonitrile in 0.1% (v/v) TFA for 3 min, followed by a linear increase of the acetonitrile component from 3 to 41% in 11 min (total run time of 14 min).

NEP Degradation Trials

Insect kinin analogs (12.5 μM) were incubated at 35°C with 10 ng human recombinant NEP (a gift from Dr. A.J. Kenny, School of Biochemistry and Molecular Biology, University of Leeds) in 0.1*M* Tris-HCl, pH 7.5 for 30 min. The reaction was stopped by the addition of TFA to a final concentration of 2.7% (v/v) and the volume was made up to 250 μl with 0.1% (v/v) TFA before HPLC analysis. HPLC analysis of the fragments was performed using a Pharmacia Pep-S column and a linear gradient of acetonitrile (5–36%) in 0.1% TFA, at a flow rate of 0.8 ml/min. The peptides and fragments were monitored by a UV detector set at 214 nm. Rates of hydrolysis were calculated from the percentage decline of the substrate, as measured by changes in peak height, and in comparison with a substrate standard treated under the same conditions, but without enzyme. More experimental detail is presented in a previous manuscript.[13]

RESULTS

With an EC_{50} value of 30 p*M*, Ac-RFF[**β^3Pro**]WGa (**1460**) proved to be the most potent of the β-amino acid insect kinin analogs in stimulation of fluid secretion on the isolated Malpighian tubules of the cricket *Acheta domesticus*, at the higher end of the potency range of the natural achetakinins (20–325 p*M*). Double-replacement analog Ac-R[**β^3Phe**]-FF[**β^3Pro**]WGa (**1577**) and single-replacement analog Ac-R[**β^3Phe**]FPWGa (**1457**) proved to be somewhat less potent, with EC_{50} values of 100 and 270 p*M*, respectively, although both values remain within the potency range of the insect kinins native to the cricket. Double-replacement analog Ac-RF[**β^3Phe-β^3Pro**]WGa (**1578**) was about 3 times less active in this assay than **1460** with an EC_{50} value of 1000 p*M*, falling just outside of the potency range of the achetakinins. Single-replacement analogs Ac-R[**β^2homoPhe**]FPWGa (**1458**), Ac-RFFP[**β^3Trp**]Ga (**1459**), and Ac-RFFP[**β^2Trp**]Ga (1656) demonstrated significantly lower potency with EC_{50} values of 22,500, 20,000, and 40,000 p*M*, respectively (Table I). Finally, double-replacement analog Ac-R[**β^3Phe**]F[**β^3Pro**]WGa (**1552**) proved to be about 5700 times less potent than **1460**, with an EC_{50} of 170,000 p*M* (Table I). Nonetheless, all analogs produced a maximal diuretic response that was not significantly different from that obtained with the endogenous achetakinin peptides.[4,8]

As these peptide analogs feature modifications of residues that flank the two known peptidase-hydrolysis sites of the

Table I Cricket Malpighian Tubule Secretion Activity of β-Amino Acid Containing Insect Kinin Analogs and Naturally Occuring Achetakinins

		Cricket (*Acheta domesticus*) Malpighian Tubule Fluid Secretion	
	Peptide Analog	EC_{50} (pM)[a]	Maximum Response (%)
	Natural AK Activity Range	20–325	100
1460	Ac-Arg-Phe-Phe-[**β^3Pro**]-Trp-Gly-NH_2	30 (10–250)	110
1457	Ac-Arg-[**β^3Phe**]-Phe-Pro-Trp-Gly-NH_2	270 (110–720)	100
1459	Ac-Arg-Phe-Phe-Pro-[**β^3Trp**]-Gly-NH_2	20,000 (7,500–53,500)	100
1458	Ac-Arg-[**β^2homoPhe**]-Phe-Pro-Trp-Gly-NH_2	22,500 (7,900–63,900)	95
1656	Ac-Arg-Phe-Phe-Pro-[**β^2Trp**]-Gly-NH_2	40,000 (23,600–67,700)	97
1552	Ac-Arg-[**β^3Phe**]-Phe-[**β^3Pro**]-Trp-Gly-NH_2	170,000 (60,000–470,000)	100
1577	Ac-Arg-[**β^3Phe**]-Phe-Phe-[**β^3Pro**]-Trp-Gly-NH_2	100 (40–280)	100
1578	Ac-Arg-Phe-[**β^3Phe**]-[**β^3Pro**]-Trp-Gly-NH_2	1000 (500–1,900)	100

[a] 95% confidence limit (CL) values in parentheses.[10]

Table II β-Amino Acids Containing Insect Kinin Analogs—Enhancement of Peptidase Resistance

		% Hydrolysis	
	Peptide Analog	Human NEP	*Drosophila* ACE
LK I	DPA-Phe-Asn-Ser-Trp-Gly-NH_2	99.8	81.6
1460	Ac-Arg-Phe-Phe-[**β³Pro**]-Trp-Gly-NH_2	15.4	10.7
1552	Ac-Arg-[**β³Phe**]-Phe-[**β³Pro**]-Trp-Gly-NH_2	0	10.9
1577	Ac-Arg-[**β³Phe**]-Phe-Phe-[**β³Pro**]-Trp-Gly-NH_2	0	3.5
1578	Ac-Arg-Phe-[**β³Phe**]-[**β³Pro**]-Trp-Gly-NH_2	2	16.5

insect kinins[3] (Figure 1), they were evaluated for susceptibility to endopeptidase hydrolysis by *Drosophila* ACE and human NEP. Only 11% of the most active single-replacement, β-amino acid insect kinin analog **1460** (Ac-RFF[**β³Pro**]WGa) was hydrolyzed by ACE from the fruit fly *Drosophila melanogaster* over 2 h, an incubation period sufficient to hydrolyze most (82%) of the natural insect kinin leucokinin I (LK I) (Table II).[8] The sequence of LK I is Asp-Pro-Ala-Phe-Asn-Ser-Trp-Gly-NH_2, which shares with achetakinin IV (AK IV) an Asn residue in the variable X position of the C-terminal pentapeptide core region.[8] ACE also has been shown to hydrolyze the insect kinins LK II (DPG-Phe-Ser-Ser-Trp-Gly-NH2), Cus-DP I (QP-Phe-His-Ser-Trp-Gly-NH_2), Cus-DP II (NNANV-Phe-Tyr-Pro-Trp-Gly-NH_2), and Cus-DP III (SKYVSQK-Phe-Phe-Ser-Trp-Gly-NH_2).[12] It should be noted that Cus-DP II contains a Pro residue in the third position of the pentapeptide core region, as do three of the five achetakinins.[8] Over the 30 min time period in which 100% of LK I was degraded, this same analog (**1460**) also demonstrated enhanced resistance to hydrolysis by the endopeptidase NEP, with only 15% degradation observed (Table II).

The double-replacement β-amino acid insect kinin analogs **1552** (Ac-R[**β³Phe**]F[**β³Pro**]WGa) and **1578** (Ac-RF[**β³Phe-β³Pro**]WGa) demonstrated degradation levels similar to single-replacement analog **1460** upon challenge with ACE, with observed hydrolysis rates of only 11% and 16%, respectively (Table II). However, a marked improvement in resistance to ACE was observed for the double-replacement analog **1577** (Ac-R[**β³Phe**]FF[**β³Pro**]WGa), in which only 3–4% was hydrolyzed. Notably, essentially complete resistance to degradation by NEP was observed for all three double-replacement analogs (Table II).

DISCUSSION

Despite the incorporation of an additional methylene group (—CH_2—) within the backbone of the C-terminal pentapeptide core region, single replacement of amino acids with their β-amino acid counterparts (Figure 2) led to significant retention of biological activity in an in vitro insect diuretic assay. The most potent (Table I) single-replacement analog **1460** (Ac-RFF[**β³Pro**]WGa) (EC_{50} = 30 p*M*), featured modification of the Pro in the 3rd position of the pentapeptide core region. Modifications of the Phe^1 and Trp^4 residues (**1457**, **1458, 1656**, and **1459**) led to more severe losses in biological potency (Table I). The observed order of potency is consistent with the currently accepted receptor interaction model presented in Figure 3, in which the insect kinin pentapeptide core adopts a β-turn over the four core residues Phe^1-Xxx^2-Pro/Ser^3-Trp^4 (in these analogs, Xxx^2 = Phe). In this receptor model, the two critical residues Phe^1 and Trp^4 associate together in an electrostatic fashion and form an aromatic surface that directly interacts with the receptor site. The variable Xxx^2 position and the Pro/Ser^3 are depicted as pointing away from the receptor surface, with direct interaction with the receptor occurring to a much lesser extent. Clearly the **β³Pro** of the potent analog **1460** is compatible with the β-turn at this position, and its high activity is likely due to the fact that changes at this position have a relatively small impact on the interaction of the analog with the receptor site.

The most potent analog **1460**, featuring a β-amino acid replacement for the Pro^3 residue of the core, was utilized as a starting point to design and synthesize double-replacement analogs, which could potentially extend protection to the secondary site of peptidase susceptibility (Figure 1). The receptor-interaction model (Figure 3) also helps to explain the relative potencies of the double-replacement analogs **1552** (Ac-R[**β³Phe**]F[**β³Pro**]WGa), **1577** (Ac-R[**β³Phe**]FF[**β³Pro**]-WGa), and **1578** (Ac-RF[**β³Phe-β³Pro**]WGa). In the most potent double-replacement analog **1577** (EC_{50} = 100 p*M*), which falls within the potency range of the natural insect kinins, the additional β-amino acid is placed adjacent to the secondary peptidase-susceptible peptide bond and just *outside* of the pentapeptide core region. The additional β-amino acid therefore does not greatly interfere with interaction of the analog with the receptor site. In the double-replacement analog **1552**, the other flank of the secondary peptidase-susceptible site is protected by incorporation of a β-amino

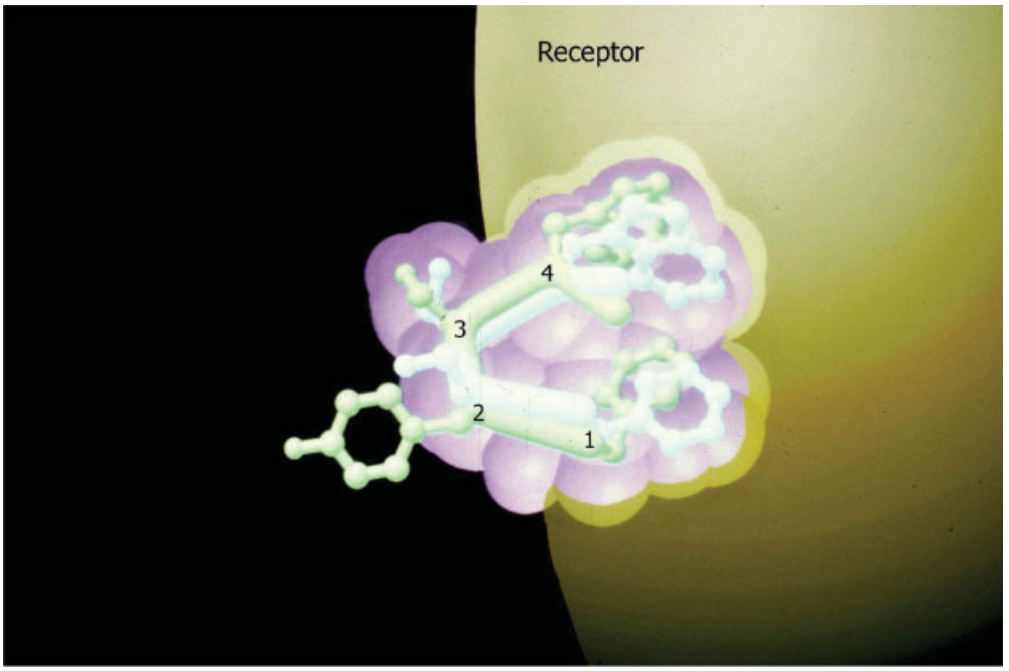

FIGURE 3 The currently accepted receptor-interaction model of an insect kinin C-terminal pentapeptide core region in a 1–4 turn, the active conformation.[5,6,8,9,11]

acid; however, this occurs at the critical Phe[1] residue. The additional β-amino acid does interfere with the interaction of the analog with the receptor site, and leads to a severe loss of biological activity (EC_{50} = 170,000 p*M*). In the second most active (EC_{50} = 1000 p*M*) double-replacement analog **1578** (Ac-RF[***β*^{3}Phe-*β*^{3}Pro**]WGa), the additional β-amino acid is placed at the variable Xxx[2] position that points away from the receptor site[6,8,9] (Figure 3) and it therefore interferes with the interaction of the analog with the receptor site to a lesser extent than when present in a critical position.

Although the flexibility of a number of the β-amino acid-containing analogs does not impede interaction with the insect kinin receptors on the cricket Malpighian tubule, the structural modification does impede hydrolysis of the analogs by the endopeptidases ACE and NEP. Presumably, the extended main chain of the β-amino acid residues (Figure 2) interferes with successful binding with the enzyme. The protection of the N-terminus with an Ac group confers resistance to aminopeptidase attack as well.[17] The endopeptidase ACE is responsible in mammals for the conversion of angiotensin I to angiotensin II and the inactivation of bradykinin.[1,18,19] Other mammalian hormones such as cholecystokinin,[20] (Leu[5])enkephalinamide, and (Met[5]) enkephalinamide,[21] substance P, and LH-RH are substrates of, and are inactivated by, ACE. Similarly in insects, a number of naturally occurring peptides implicated in the control of water and ion balance, development, digestion, and visceral muscle contraction have proven to be substrates of insect ACE.[12] Soluble ACE has been found in the hemolymph of insects,[10–13] where the enzyme may have a role in regulating levels of circulating peptide hormones by inactivating them through hydrolysis of the critical C-terminal region. In our experiments, 82% of the natural analog LK I is degraded over a 2 h period.

Another peptidase found in insects, the endopeptidase NEP, does have broad specificity, preferentially hydrolyzing peptides by cleaving on the N-terminal side of hydrophobic amino acids such as Phe, and sometimes Trp.[22,23] The natural LK I analog was readily hydrolyzed with a dose of 10 ng of NEP, and was largely degraded within 30 min. Conversely, the β-amino acid containing analogs demonstrated greatly enhanced stability to both ACE and NEP endopeptidases. With the single-replacement analog **1460** (Ac-RFF[***β*^{3}-Pro**]WGa), greatly reduced hydrolysis rates of 15% and 11% were observed for NEP and ACE, respectively (Table II). Double-replacement analogs **1552** (Ac-R[***β*^{3}Phe**]F[***β*^{3}Pro**]-WGa) and **1578** (Ac-RF[***β*^{3}Phe-*β*^{3}Pro**]WGa) demonstrated similar hydrolysis rates of 11 and 16%, respectively. However, double-replacement analog **1577** (Ac-R[***β*^{3}Phe**]FF[***β*^{3}Pro**]WGa) featured a markedly improved hydrolysis rate of only 3–4%. Notably, all three double-replacement analogs were essentially completely resistant to degradation by NEP under the experimental conditions. It is interesting to note that additional β-amino acid in analog **1578** (Ac-RF[***β*^{3}Phe-*β*^{3}Pro**]WGa) confers enhanced resistance to NEP, despite the

fact that it is not placed adjacent to the secondary peptidase-susceptible site. In consideration of both biological potency and enhanced resistance to peptidase degradation, the best analogs would appear to be double-replacement analog **1577** (Ac-R[**β^3Phe**]FF[**β^3Pro**]WGa), single-replacement analog **1460** (Ac-Arg-Phe-Phe-[**β^3Pro**]-Trp-Gly-NH_2), and double-replacement analog **1578** (Ac-RF[**β^3Phe-β^3Pro**]WGa).

CONCLUSIONS

Incorporation of β-amino acid residues into insect neuropeptides can produce potent analogs that are more flexible and may therefore retain an ability to interact with the receptor despite the structural modification. In addition, this modification can lead to analogs that are more resistant to enzyme hydrolysis. These results open additional possibilities for the design of potent agonists/antagonists, which could provide the basis for selective, environmentally friendly pest arthropod control strategies based on insect kinin neuropeptides.

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